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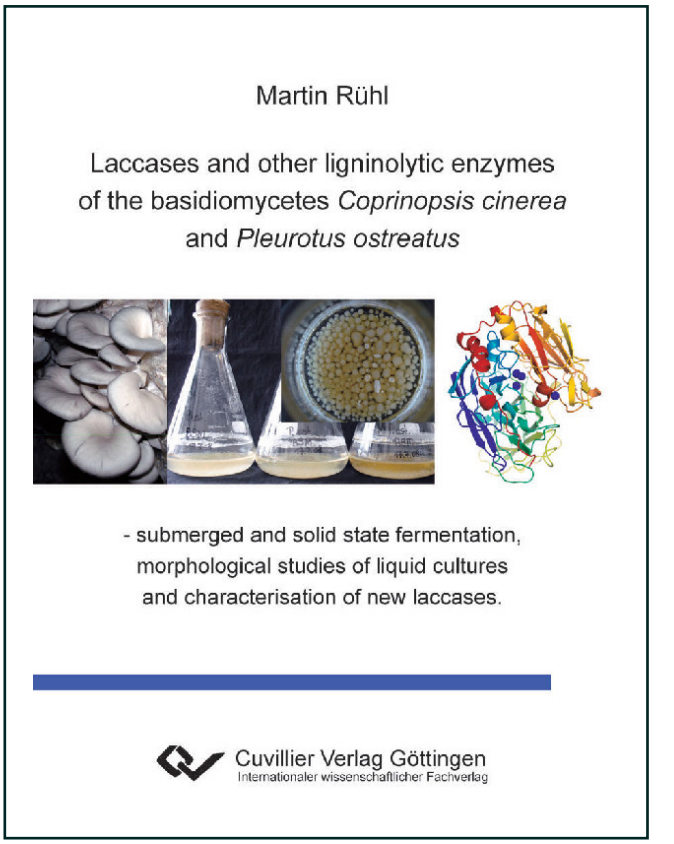
Laccases and other ligninolytic enzymes of the basidiomycetes Coprinopsis cinerea and Pleurotus ostreatus

- submerged and solid state fermentation, morphological studies of liquid cultures and characterisation of new laccases



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Chapter 1

Introduction

I. Laccases: sources and applications

II. Basidiomycetes: higher fungi of great economical and ecological value

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http://webdoc.sub.gwdg.de/univerlag/2007/wood_production.pdf

III. Aim of the thesis

IV. References

I. Laccases: sources and applications

The widespread enzyme laccase (EC 1.10.3.2, benzenediol:oxygen oxidoreductase) produced by many ligninolytic fungi belongs to the family of multi-copper enzymes (Messerschmidt et al. 1989) that oxidise a wide range of substrates through a one-electron oxidation reaction. In general, laccases contain four copper atoms distributed in three copper sites (T1, T2 and T3). The electron migration between the substrate and the enzyme is regulated by the T1 site, also known as blue copper site (Solomon et al. 1996), and, thus, depends on the redox potential (= reduction and oxidation potential) of this site. In fungal laccases, oxidation potentials of the blue copper can vary e.g. between 0.46 V and 0.79 V as determined for *Myceliophthora thermophila* and *Trametes versicolor*, respectively (Xu 1996; chapter 5). Electron migration in laccases takes place via a cysteine-hystidine pathway from the T1 site to the T2 and T3 sites. T2 and T3 form a trinuclear copper cluster, with one copper atom at the T2 site and two copper atoms at the T3 site, where dioxygen is reduced to water (Solomon et al. 1996, Ducros et al. 1998, Messerschmidt 1998, Hakulinen et al. 2002, Piontek et al. 2002).

Natural organic substrates, such as phenolic compounds, can be attacked directly by laccases with subsequent generation of phenoxy radicals (Fig. 1A), which can perform radical coupling or substitution reaction with other molecules (Solomon et al. 1996). In cases where physical (size) or chemical (redox-potential) properties of the laccase restrict an oxidation of the substrate, small mediating substances with higher oxidation potentials called mediators may be used (d'Acunzo et al. 2006, Fig. 1B). Mediators are activated by laccases via a monoelectronic oxidation and can afterwards react with substrates, which are not able to be degraded by laccases alone. Thus, non-phenolic substances may be degraded by laccases by usage of different kinds of natural [phenols, aniline, 4-hydroxybenzoic acid (HBA), 4-hydroxybenzyl alcohol, cysteine, methionine] and non-natural [2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1-hydroxybenzotriazole (HBT), N-hydroxyacetanilide (NHAA), 2,2,6,6-tetramethylpiperidin-1-yloxy (TEMPO) and violuric acid (VA)] mediators (Johannes and Majcherczyk 2000, Camarero et al. 2005). The combination of a laccase and a suitable mediator is known as laccase mediator system (LMS). Such systems are used in different industries (section 1.I.B) and may also be found in nature, where suitable mediators enable the fungal laccases to degrade recalcitrant substances (Johannes and Majcherczyk 2000).

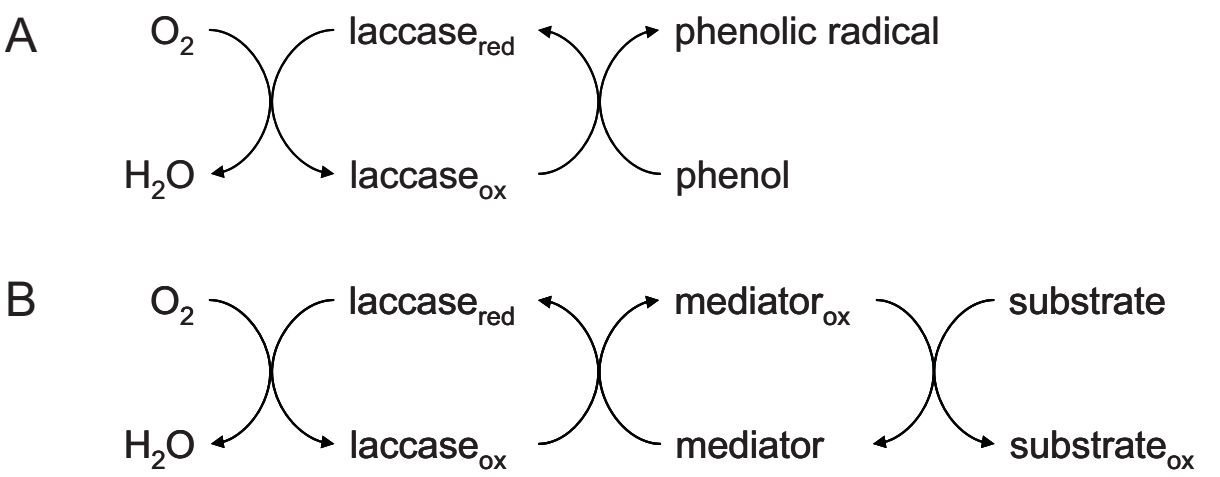

Fig. 1 Schematic reaction cascade of a laccase with a phenol (**A**) and of a laccase mediator system (**B**) after Bourbonnais et al. (1998).

A. Occurrence and functions of laccases in nature

In general, laccases are produced by various microorganisms (certain bacteria and mainly fungi) (Claus 2004), but they are also found in plants (Andreasson et al. 1976, Richardson et al. 2000, Gavnholt and Larsen 2002), insects (Sugumaran et al. 1992, Kramer et al. 2001, Arakane et al. 2005) and even crustaceans (van der Ham and Felgenhauer 2007). In the latter cases, laccase is needed for melanisation/tanning processes as well as in the immune response and wound healing (Decker et al. 2001, Lee and Soderhall 2002, Sugumaran 2002, Liu et al. 2006, Van Der Ham and Felgenhauer 2007). Insects use laccases also for tanning and for cuticle sclerotisation (Kramer et al. 2001) while in plants laccases are involved in the polymerisation of lignin (O'Malley et al. 1993).

In bacteria, laccases or laccase-like multicopper oxidases (LMCOs) were found in gram-positive bacteria, such as *Bacillus licheniformis* (Koschorreck et al. 2008), and in *Escherichia coli* (Kataoka et al. 2007), *Pseudomonas syringae* (Cha and Cooksey 1991) as well as in other gram-negative bacteria (e.g. Pseudomonadaceae) (Sharma et al. 2007). In these microorganisms, laccases might be involved in a multiplicity of processes, such as pigmentation, Cu^{2+} resistance, Mn^{2+} oxidation, sporulation and others (Sharma et al. 2007).

Most laccases are of fungal origin, where they are found in ascomycetes and basidiomycetes (Hoegger et al. 2006). Usually, laccases are extracellular proteins secreted by the fungi for oxidation reactions outside the cell. They occur in the soil inhabiting ascomycetous fungus *Aspergillus nidulans* during the sexual (Hermann et al. 1983, Scherer and Fischer 1998) as well as during the asexual life cycle (Aramayo and Timberlake 1990 and 1993, Scherer and

Fischer 1998). Another soil inhabiting deuteromycete fungus *Pestalotiopsis* sp. showed high natural laccase activity, which could be further increased in liquid culture (Hao et al. 2007). Laccase genes and activity are also found in the ascomycete family *Morchellaceae*, where saprotrophic, mycorrhizal-like and faint parasitism growth was observed (Kellner et al. 2007). In the ascomycetous plant pathogenic fungi *Gaeumannomyces graminis* var. *tritici* (Thompson et al. 2006) and *Fusarium proliferatum* (Anderson et al. 2005), laccase activity was detected as well. Furthermore, laccases have been found in thermophilic fungi such as the ascoymcetes *Myceliophthora thermophila* and *Chaetomium thermophilum* (Berka et al. 1997, Chefetz et al. 1998, Maheshwari et al. 2000). Laccases occur as well in fungal symbiosis such as in lichens. There, species of the sub-order *Peltigerineae* showed higher activities (mean 30fold) compared to other sub-orders, such as *Lecanorineae*, where low or no laccase activity was detected (Laufer et al. 2006). It is most probable that in these symbiotic fungal associations, laccases are involved in the metabolism of secondary phenolic compounds, like lichen acids (Lisov et al. 2007). In a pathogenic association community related to the esca syndrome, a severe illness in vine plants affecting all open plant parts, (ascomycetes: *Phaeomoniella chlamydospora* and *Togninia minima*, basidiomycete: *Fomitiporia mediterranea*) all three fungi produce laccases (Bruno and Sparapano 2006). The authors believe that the white-rot basidiomycete *F. mediterranea* laccase is involved in lignin metabolisation, whereas the ascomycetous laccases are important for the detoxifation of a polyphenolic compound (resveratrol) acting as antioxidant in grapes. Furthermore, laccase activity was detected in liquid shaken cultures of *Monotospora* sp., an endophytic fungus of the grass *Cynodon dactylon* (Wang et al. 2006).

While laccases are present throughout the fungal kingdom (Hoegger et al. 2006), basidiomycetes are the most common source for this enzyme under natural growth conditions. In ectomycorrhizal (EM) basidiomycetous fungi, noticeable laccase activity was detected in two *Lactarius* and all *Rusulla* species tested by Gramss et al. (1998). However, the highest extracellular laccase activity was detected in litter degrading species tested also in this work. The enzyme activity in the EM fungi seemed to be basically intracellular, whereas the litter degrading fungi showed predominantly extracellular activity (Gramss et al. 1998).

In another study of EM fungi, laccase activity was found on the tips of *Lactarius quietus* and *Cortinarius anomalus* in oak forests (Courty et al. 2006). Highest activities occurred during spring time, which can be ascribed to the degradation of the organic matter in the soil. This process takes place either to possibly obtain carbon (C) for the saprotrophic growth of the

fungi or to deliver nitrogen (N) for the vegetative growth of the trees (Courty et al. 2006). It is particularly interesting that the EM species *Laccaria bicolor* has 9 different laccase genes of which 3 are highly expressed in ectomycorrhizas, 1 in fruiting bodies of *L. bicolor* and 1 in mycelium of *L. bicolor* grown on glucose rich agar medium (Courty et al. 2009).

From results of a comparative study of five different basidiomycetes, of which three are litter degrading and two white-rot fungi, laccase was stated to be the most frequent ligninolytic enzyme in litter degrading fungi (Baldrian and Snajdr 2006). Contradictory, Ullah et al. (2002) tested several leaf litter decomposers and wood degraders and found that, overall, the ligninolytic enzyme manganese peroxidase MnP (EC 1.11.1.13, MnP, Mn(II):hydrogen-peroxide oxidoreductase) activity was higher in litter decomposers, whereas in wood decaying fungi the laccase activity was higher (Gregorio et al. 2006). In general, the wood degraders can be classified into brown- and white-rot fungi, of which only the latter ones are capable of degrading the lignin and, therefore, having a ligninolytic enzymatic system (Hoegger et al. 2007). Nevertheless, laccases were also found in brown-rot fungi, like *Gloeophyllum trabeum* and *Postia placenta* (D'Souza et al. 1996). A recent analysis of the established genome of *P. placenta* detected 5 MCO (multicopper oxidase) genes, of which 3 are obviously for laccases (Martinez et al. 2009). Contradictory, one typical representative of the white-rot fungi, *Phanerochaete chrysosporium*, has 4 *MCO* genes whose products are not laccases (Martinez et al. 2004). This emphasises the finding that *P. chrysosporium* is lacking classical laccase activity (Larrondo et al. 2003).

The basic ligninolytic system of fungi consists of laccase, MnP and lignin peroxidase (EC 1.11.1.14, LiP, 1,2-bis(3,4-dimethoxyphenyl)propane-1,3-diol: hydrogen-peroxide oxidoreductase), which are produced in different combinations. Hatakka (1994) divided the white-rot fungi into 3 classes, according to the enzymes which are primarily involved in the degradation of lignin: LiP and MnP, MnP and laccase, and LiP and laccase. However, several studies show that certain fungi produce all three types of enzymes and other fungi exist where only laccase activity is detected (Table 1). The classification Hao et al. (2006) offers would comprise all possible groups: With the classes I, II, III and IV, respectively, producing only LiP and MnP (I), all three types of enzymes (II), laccase with either LiP or MnP (III) and solely laccase (IV). However, some white-rot fungi are difficult to classify in this system, as a lack of enzymatic activities under tested growth conditions does not indicate whether the enzymes might not be produced under altered conditions. The saprotrophic dung fungus *Coprinopsis cinerea* has 17 different laccase genes which are differently expressed under altered cultivation parameters such as media and temperature (Schneider et al. 1999,

Navarro-González 2008, section 4.I). However, the production of ligninolytic enzymes of species such as *C. cinerea* and *Ganoderma lucidum* seems also to be strain specific (Silva et al. 2005a, Table 1 and section 4.I). No MnPs or LiPs are present in *C. cinerea, L. bicolor* and *P. placenta*, but all of them seem to have another type of low redox peroxidase not closely related to MnP and LiP (Martinez et al. in 2009). Recently available genomes of the white-rot fungi *P. ostreatus* and *Schizophyllum commune* will show which genes coding for ligninolytic enzymes are present in these fungi and, thus, help to clarify the role of these enzymes in lignin degradation.

Table 1 Ligninolytic enzyme activities of a variety of basidiomycetes where laccase activity was detected

		Enzymes			
Fungal species		**MnP**	**LiP**	**Lac**	**Reference(s)**
Agaricus bisporus		+	ND*	+	Bonnen et al. 1994
Bjerkandera adusta		+	+	+	Hatakka 1994
Cerioporiopsis subvermisopra		+	-	+	Rüttimann et al. 1992
Collybia sp.		+	+	+	McErlean et al. 2006
Coriolopsis polyzona		+	-	+	Nerud et al. 1991
Dichomitus squalens		+	-	+	Nerud et al. 1991
Fomes sclerodermus		+	ND	+	Papinutti and Martinez 2006
Ganoderma australe		-	-	+	Elissetche et al. 2006
Ganoderma lucidum		+	ND*	+	D'Souza et al. 1999, Wang and Ng 2006b
Ganoderma	CB364	-	-	+	Silva et al. 2005a
spp.	GASI3.4	+	+	+	
	CCB209	+	+	+	
	GASI2	-	-	+	
Ganoderma valesiacum		+	-	+	Nerud et al. 1991
Grammothele subargentea		-	-	+	Saparrat et al. 2008
Lentinula edodes		+	+	+	Hatakka 1994, Silva et al. 2005b
Marasmiellus troyanus		+	ND	+	Gregorio et al. 2006
Marasmius quercophilus		+	ND	+	Steffen et al. 2007a
Mycena inclinata		+	ND	+	Steffen et al. 2007a
Panus tigrinus		+	-	+	Bonnen et al. 1994
Phlebia brevispora		+	+	+	Perez and Jeffries 1990
Phlebia floridensis		+	+	+	Arora and Gill 2005
Phlebia ochraceofulva		-	+	+	Hatakka 1994
Phlebia radiata		+	+	+	Hatakka et al. 1991
Phlebia tremelosa		+	+	+	Hatakka 1994
Pholiota lenta		+	ND	+	Steffen et al. 2007a
Pleurotus ostreatus		+	-	+	McErlean et al. 2006, this work section 2.II
Pleurotus sajor-caju		+	-	+	Hatakka 1994,Fu et al. 1997
Pycnoporus cinnabarinus		-	-	+	Eggert et al. 1996
Rhizoctonia solani		+	+	+	McErlean et al. 2006
Rigidoporus lignosus		+	-	+	Hatakka 1994
Stereum hirsutum		+	-	+	Nerud et al. 1991
Trametes gibbosa		+	+	+	Nerud et al. 1991
Trametes hirsuta		+	+	+	Nerud et al. 1991
Trametes versicolor		+	+	+	Tanaka et al. 1999

MnP = manganese dependent peroxidase, LiP = lignin peroxidase, Lac = laccase, + = enzyme activity was detected, - = no activity detected, ND = not determined, * LiP-like genes are present.